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Production of Bioethanol from Rice Straw using Cellulase by Local *Aspergillus* sp.

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ABSTRACT

Cellulase production *in situ* was considered as one of the alternatives to reduce bioethanol production cost. In this study, cellulase enzyme was produced from rice straw by locally isolated *Aspergillus* sp. in solid state fermentation. The crude cellulase was measured to have activity of 6.3 FPU g⁻¹ rice straw. The rice straw was pretreated by few cycles of wet disc milling prior saccharified it using the crude cellulase produced. More than 90% glucose from total cellulose was released by the saccharification. The saccharified product was subjected to fermentation by yeast. The highest bioethanol yield produced from the fermentation was 0.102 g g⁻¹ rice straw which is equivalent to 62.61% of theoretical bioethanol yield. It was concluded that the use of crude cellulase from rice straw onto rice straw can lead to a good yield of bioethanol, provided an effective pretreatment was used.

Key words: Rice straw, crude cellulase, saccharification, fermentation, bioethanol

INTRODUCTION

Paddy is included as one of Malaysian major crop and producing huge amount of rice straw as solid biomass waste seasonally. The rice straw came after the stripping process of rice using machine at the field, where the rice straw was removed and left to dry. It has no further use apart from being used as fodder. Since, it exists in abundant amount, it is usual for farmers to burn the rice straw when it dried at their field, causing haze and other environmental problem (Lai *et al.*, 2009). It is also usual for them to throw it away if the rice straw is wet due to rain. Both conventional elimination methods definitely cause environmental pollution. With regard to depleting fossil fuel issue, there are biotechnological approaches to utilize rice straw as alternative fuel, for example bioethanol (Binod *et al.*, 2010).

Bioethanol was a term referring to ethanol produced from cellulosic biomass (Mabee and Saddler, 2010). Currently, bioethanol is commonly used as fuel additive and industrial chemical product (Hernandez and Kafarov, 2007). Biomass such as rice straw contains three major components which are cellulose, hemicellulose and lignin. Accordingly, only cellulose and hemicellulose have the ability to be converted into sugars (Moiser *et al.*, 2005). Cellulose is a polymer constructed by chains of D-glucose linked by $\beta(1-4)$ bond. This bond can be broken by introducing external chemical such as acid, or catalyze by enzymatic hydrolysis (Karimi *et al.*, 2006a). Usage of acid for rice straw hydrolysis is considered as non-green approach since the

discharge will be non-environmental-friendly and it is costly to treat the waste at large scale (Sun and Cheng, 2002). Therefore, the use of cellulase enzyme as the catalytic agent is more favorable, but the use of commercial enzyme is costly.

It was suggested that enzyme should be produced *in situ* and used in crude form to reduce the cost (Fang *et al.*, 2009). There are few studies on cellulase production from raw biomass such as rice straw and empty fruit bunches, but it is difficult and expensive to produce enzyme with high activity which is the only drawback. In this study, we targeted to produce high activity cellulase enzyme from rice straw and use it in crude form on thermal treated wet disc milled rice straw. The performance of bioethanol fermentation on the saccharification product was also studied. Profile of the saccharification product and bioethanol fermentation was studied. Additionally, we also studied effect of thermal treatment after disc milling treatment on the yield of bioethanol produced.

MATERIALS AND METHODS

Substrate: The rice straw was obtained from Sekinchan paddy field (Selangor, Malaysia) on August 2009. It was sundried and ground to 2 mm using hammer mill grinder. Ground rice straw was kept in cold room at 4°C until further use. Proximate analysis showed that the rice straw consist of 32.01% cellulose, 26.98% hemicellulose and 22.66% of lignin.

Pretreatment of rice straw: Alkaline and thermal treatment was used on rice straw for enzyme production only. Ground rice straw was subjected to combination of chemical and thermal treatment as per method described by Roslan *et al.* (2009). Rice straw was submerged in 0.5% NaOH (with ratio of rice straw to NaOH solution of 1:10) and heated at 121°C for 20 min. It was then washed with tap water until the pH stable at nearly neutral and rinsed with distilled water before oven dried at 60°C overnight.

Inoculum preparation: Fungus *Aspergillus* sp. was obtained from Biomass Technology Centre laboratory collection while *Saccharomyces cerevisiae* ATCC 24860 was bought from ATCC. Fungus spores and yeast cells were kept in 30% glycerol at -20°C. Fungus spores were reactivated by inoculation on Potato Dextrose Agar (PDA) for 7 to 9 days. Spores collection were performed by washing the agar surface with sterile distilled water prior to spore counting using heamocytometer. Yeast cells were reactivated by inoculation into yeast, peptone, D-(+)-glucose broth (YPD) for 24 h and rinsed two times using sterile saline water and centrifuged to remove excessive water.

Cellulase fermentation and extraction: Solid state fermentation method was used since it consumes lesser power but produce more concentrated product. Cellulase production was carried out in solid state fermentation using modified method from Roslan *et al.* (2009). A series of 10 Erlenmeyer flasks were used in this experiment to produce cumulative volume of cellulase. Three grams of pretreated rice straw was inserted into each 250 mL Erlenmeyer flask with cotton stopper. Modified Mandel's medium (1 L of Modified Mandels contained 1.4 g (NH₄)₂SO₄, 2.0 g KH₂PO₄, 0.3 g CaCl₂, 0.3 g MgSO₄·7H₂O, 1 mL of trace element and 50 mL of palm oil mill effluent, POME) was used as supplement where 60% (6 mL g⁻¹ substrate) of the solution was added into the flask. Each flask was finally inoculated with 1×10⁶ of fungal spores (*Aspergillus* sp.) and incubated at 30°C for 7 days. Cellulase extraction was done by adding 30 mL of 50 μM acetate buffer into each flask. It was then immersed for 30 min prior centrifuged at 3000 rpm for 15 min and filtered. Enzyme activity was determined using (Wood and Bhat, 1988). Supernatant were kept at 4°C prior use or stored at -20°C. The crude cellulase was used in enzymatic hydrolysis.

Enzymatic hydrolysis: Rice straw used as substrate in this study was treated using wet disc milling method and no chemical (acid nor alkaline) was used. Wet disc milling and enzymatic hydrolysis was performed using modified hydrolysis method from Hiden *et al.* (2009) using crude cellulase produced by *Aspergillus* sp., in this experiment. Wet disc milled rice straw was divided into two portion where one of them was treated with thermal. Ten g of wet disc milled rice straw slurry (contained 1 g of rice straw in dry weight) with 6 FPU of crude cellulase in 50 mM acetate buffer at pH 4.8 was added into 50 mL tube with magnetic bar. Final volume was adjusted to 30 mL using acetate buffer. It was incubated at 50°C for 72 h with agitation and 1 mL of sample was taken at time 0, 24, 48 and 72 h. This experiment was done in triplicate. Sugar analysis was done using HPLC (Jasco, RI 1530). Sugar yields were calculated by measuring the sugar obtained based on equation below:

$$\text{Sugar (\%)} = \left(\frac{\text{mg glucose obtained}}{\text{Total potential glucose}} \right) \times 100\%$$

Bioethanol fermentation: Bioethanol fermentation was conducted in liquid state fermentation. Yeast was pregerminated in YPD media at the same volume with the actual fermentation medium prior inoculation. This is to ensure the yeast is ready to work at full performance in the fermentation medium. Bioethanol fermentation was carried out using saccharified rice straw from enzymatic hydrolysis. The pH was modified from 5.0 to 6.0 prior to addition of yeast cells in saline water to avoid sugar inhibition (Linden *et al.*, 1992). All tube was incubated at 30°C for 48 h with agitation. Sample was taken at 0, 24 and 48 h for analysis. Ethanol concentration was measured using GC (Shimadzu 17A) with capillary column BP-21. The GC condition was as follows: column temp 40°C, injector 150°C, detector 200°C. The column was allowed to rest at 40°C for 1 min before increasing the temperature by 20°C min⁻¹ and then it was held for 1 min at 130°C. After that the GC was allowed to cool down.

RESULTS AND DISCUSSION

Cellulase production, activity and enzymatic hydrolysis: All cellulase produced in solid state fermentation was collected in a single container where the cumulative activity was determined to be at 6.3 FPU 10 mL⁻¹. Six FPU of cellulase was used in each flask for saccharification throughout the study. From Table 1, it was observed that glucose produced from saccharification process by non-thermal treatment disc milled rice straw ranged from 0.221-0.245 g g⁻¹ rice straw. This value counted about 69.01-76.56% of total cellulose. However, for thermal treated rice straw, it was observed that glucose was produced at higher concentration and rate, ranged from 0.308-0.380 g g⁻¹ rice straw. It was suggested that the additional of thermal treatment on disc milled rice straw facilitated enzymatic hydrolysis due to creation of amorphous region in starch molecules when it was heated. Additionally, heating up also made the water in the medium in a partially acidic due to the release of acetyl group from hemicellulose. This causing the medium to be slightly acidic, which explain the increase in saccharification (Bacon *et al.*, 1981). These value however were slightly higher than total cellulose percentage and it was suggested that some of the glucose came from hemicellulose due to enzymatic activity of hemicellulase in the crude enzyme. It is uncertain on how much glucose was contributed by hemicellulose. However, it was confirmed that this cellulase enzyme, which was produced from rice straw, was highly suitable for use with

Table 1: Glucose produced by saccharification on different treatments of rice straw. Measured as gram glucose produced from gram rice straw

Sample	Glucose concentration (g glucose g ⁻¹ rice straw)			
	0 h	24 h	48 h	72 h
DM 5 cycle	0.028	0.131	0.115	0.225
DM 10 cycle	0.024	0.198	0.162	0.245
DM 15 cycle	0.025	0.182	0.179	0.233
DM 20 cycle	0.025	0.102	0.198	0.221
DM 5 cycle with thermal	0.026	0.177	0.146	0.363
DM 10 cycle with thermal	0.029	0.169	0.202	0.364
DM 15 cycle with thermal	0.023	0.199	0.150	0.308
DM 20 cycle with thermal	0.027	0.233	0.158	0.380

Table 2: Percentage of ethanol yield over total potential sugar from rice straw with different treatment

Sample	Ethanol yield (%)			
	0 h	24 h	48 h	72 h
DM 5 cycle	0.00	36.94	40.13	34.80
DM 10 cycle	0.00	38.79	39.32	36.10
DM 15 cycle	0.00	38.27	36.28	34.69
DM 20 cycle	0.00	43.46	43.35	35.10
DM 5 cycle with thermal	0.00	49.98	53.29	50.08
DM 10 cycle with thermal	0.00	53.33	52.09	51.41
DM 15 cycle with thermal	0.00	46.72	47.52	47.26
DM 20 cycle with thermal	0.00	59.47	62.61	58.31

pretreated rice straw. This is due to cellulase produced from same biomass as to be used as the bioconversion substrate proved more efficient than the one produced from other cellulosic substrate (Lynd *et al.*, 2002).

Bioethanol fermentation: From Table 2, it was observed that all fermentation completed in 24 h except in few cases, where a little increment of ethanol can be seen after 48 h. It was also found that ethanol production and yield tends to increase with the increment of wet disc milling cycle and addition of thermal treatment. Given the total cellulose content in rice straw was 32.01%, theoretically 0.16 g of ethanol can be obtain if 100% conversion occur. Punnapayak and Emert (1986) obtained less than 30% of ethanol yield can be obtained using alkaline pretreated rice straw while Karimi *et al.* (2006b) obtained a maximum of 74% of ethanol yield using simultaneous saccharification and fermentation of acid pretreated rice straw. Simultaneous saccharification and fermentation of alkaline and microwave/alkaline pretreated rice straw yielded 61.3% of ethanol. Zhu *et al.* (2006) and Sukumaran *et al.* (2009) reported efficiency of 40.33 and 41.79% was achieved for ethanol conversion from rice straw with acid or alkaline with thermal pretreatment. With only disc milling without application of thermal, acid nor alkaline for the rice straw in enzymatic hydrolysis, the highest ethanol yield in this study was from 20 cycles milling (43.46%) as compared to disc milled 5 cycles (40.13%). However, this value increased drastically when thermal treatment involved. Ethanol yield for 20 cycle disc milled rice straw with thermal was improved by 19.26%, which is from 43.46 to 62.61%, as compared to 20 cycle disc milled alone.

This improvement was caused by the initial glucose concentration for disc milled with thermal was already higher than disc milled alone, render it for higher ethanol production. Furthermore,

the fermentation was directly carried out using the saccharified product including the excessive biomass. In this case, there was a chance that some sugars were trapped within the biomass, which made it missed during sampling therefore not analyzed. Method used for pretreatment in this study is an advantage over chemical pretreatment. This is because phenolic inhibitors such as furfural and hydroxymethyl furfural (HMF) were released during pretreatment by acid or during acid hydrolysis. These inhibitors have been regarded by far as the most toxic inhibitors present in lignocellulosic hydrolyzate and its presence will make the ethanol fermentation process by yeast much more complicated, rendering it increase cost of production due to detoxification process (Palmqvist and Hahn-Hagerdal, 2000).

CONCLUSION

In this study, ethanol conversion yield obtained from disc milled rice straw was higher than reported previously. This suggests that application of thermal into the treatment improve the digestibility of rice straw by enzyme. Additionally the crude cellulase obtained from this study is suitable to be used on disc milled rice straw where it leads to better yield in ethanol fermentation.

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